

(19)



Europäisches Patentamt  
European Patent Office  
Office européen des brevets



(11)

EP 0 994 190 A2

(12)

## EUROPEAN PATENT APPLICATION

(43) Date of publication:

19.04.2000 Bulletin 2000/16

(51) Int. Cl.<sup>7</sup>: C12N 15/31, C07K 14/245,

C12N 1/21, C12P 13/06,

C12P 13/08, C12R 1/19

(21) Application number: 99118581.0

(22) Date of filing: 20.09.1999.

(84) Designated Contracting States:

AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU  
MC NL PT SE

Designated Extension States:

AL LT LV MK RO SI

(30) Priority: 13.10.1998 RU 98118425

(71) Applicant: Ajinomoto Co., Inc.

Tokyo (JP)

(72) Inventors:

- Livshits, Vitaly Arkadievich  
113545 Moscow (RU)

• Zakataeva, Natalya Pavlovna

113545 Moscow (RU)

• Aleoshin, Vladimir Venyamiovich

113545 Moscow (RU)

• Belareova, Alla Valentinovna

113545 Moscow (RU)

• Tokhmakova, Irina Lvovna

113545 Moscow (RU)

(74) Representative:

Strehl Schübel-Hopf & Partner

Maximilianstrasse 54

80538 München (DE)

(54) DNA conferring L-homoserine resistance to bacteria, and its use

(57) A bacterium which has an ability to produce an amino acid and in which a novel gene (*rhtB*) coding for a protein having an activity of making a bacterium having the protein L-homoserine-resistant is enhanced, is cultivated in a culture medium to produce and accumulate the amino acid in the medium, and the amino acid is recovered from the medium.

BEST AVAILABLE COPY

EP 0 994 190 A2

**Description**Technical Field

- 5 [0001] The present invention relates to a method for producing an amino acid, especially for a method for producing L-homoserine, L-alanine, L-isoleucine, L-valine, or L-threonine using a bacterium belonging to the genus *Escherichia*.

Background Art

- 10 [0002] The present inventors obtained, with respect to *E. coli* K-12, a mutant having mutation, *thrR*, (herein referred to as *rhtA23*) that is concerned in high concentrations of threonine (>40 mg/ml) or homoserine (>5 mg/ml) in a minimal medium (Astaurova, O. B. et al., Appl. Bioch. and Microbiol., 21, 611-616 (1985)). On the basis of *rhtA23* mutation an improved threonine-producing strain (SU patent No. 974817), homoserine- and glutamic acid-producing strains (Astaurova et al., Appl. Bioch. And Microbiol., 27, 556-561 (1991)) were obtained.
- 15 [0003] Furthermore, the present inventors have revealed that the *rhtA* gene exists at 18 min on *E. coli* chromosome and that the *rhtA* gene is identical to ORF1 between *pexB* and *ompX* genes. The unit expressing a protein encoded by the ORF1 has been designated as *rhtA* (*rht*: resistance to homoserine and threonine) gene. The *rhtA* gene includes a 5'-noncoding region including SD sequence, ORF1 and a terminator. Also, the present inventors have found that a wild type *rhtA* gene participates in resistance to threonine and homoserine if cloned in a multicopy state and that enhancement of expression of the *rhtA* gene improves amino acid productivity of a bacterium belonging to the genus *Escherichia* having an ability to produce L-lysine, L-valine or L-threonine (ABSTRACTS of 17th International Congress of Biochemistry and Molecular Biology in conjunction with 1997 Annual Meeting of the American Society for Biochemistry and Molecular Biology, San Francisco, California August 24-29, 1997, abstract No. 457).
- 20 [0004] It was found that at least two different genes which impart homoserine resistance in a multicopy state exist in *E. coli* during cloning of the *rhtA* gene. One of the genes is the *rhtA* gene, however the other gene has not yet been elucidated.
- 25

Disclosure of the Invention

- 30 [0005] An object of the present invention is to provide a novel gene participating in resistance to homoserine, and a method for producing an amino acid, especially, L-homoserine, L-alanine, L-isoleucine, L-valine and L-threonine with a high yield.

- [0006] The inventors have found that a region at 86 min on *E. coli* chromosome, when cloned by a multicopy vector, impart resistance to L-homoserine to cells of *E. coli*, and the amplification of this region results, like the *rhtA* gene, in an improvement of the amino acid productivity of *E. coli*. On the basis of these findings, the present invention has been completed.
- 35

- [0007] Thus, the present invention provides:

- 40 (1) a protein comprising an amino acid sequence shown in SEQ ID No. 2 of the Sequence Listing or including deletion, substitution, insertion and/or addition of one or more amino acids in said sequence and having the activity of making a bacterium resistant to L-homoserine,
- (2) the DNA coding for a protein according to (1), which is a DNA defined in the following (a) or (b):
- 45 (a) a DNA which comprises a nucleotide sequence corresponding to the nucleotide numbers of 557 to 1171 of the nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing; or
- (b) a DNA which hybridizes with the nucleotide sequence corresponding to the nucleotide numbers of 557 to 1171 of the nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing under stringent conditions, and which codes for the protein having the activity of making the bacterium resistant to L-homoserine,
- 50 (3) a bacterium belonging to the genus *Escherichia*, wherein L-homoserine resistance of the bacterium is enhanced by amplifying the copy number or increasing the expression rate of the DNA of (2) in a cell of the bacterium,
- (4) the bacterium of (3), wherein the DNA of (1) is carried on a multicopy vector in the cell of the bacterium,
- (5) the bacterium of (3), wherein the DNA of (2) is carried on a transposon in the cell of the bacterium,
- 55 (6) a method for producing an amino acid, comprising the steps of cultivating the bacterium of any of (3) to (5), which has an ability to produce the amino acid, in a culture medium to produce and accumulate the amino acid in the medium, and recovering the amino acid from the medium, and
- (7) the method of (6), wherein the amino acid is at least one selected from the group consisting of L-homoserine,

L-alanine, L-isoleucine, L-valine and L-threonine.

[0008] The DNA of the present invention may be referred to as "*rhtB* gene", a protein coded by the *rhtB* gene may be referred to as "RhtB protein", an activity of the RhtB protein which participates in resistance to L-homoserine of a bacterium (i.e. an activity of making a bacterium having the RhtB protein L-homoserine-resistant) may be referred to as "Rh activity", and a structural gene encoding the RhtB protein in the *rhtB* gene may be referred to as "*rhtB* structural gene". The term "enhancing the Rh activity" means imparting resistance to homoserine to a bacterium or enhance the resistance by means of increasing the number of molecules of the RhtB protein, increasing a specific activity of the RhtB protein, or desensitizing negative regulation against the expression or the activity of the RhtB protein or the like.

10 The terms "DNA coding for a protein" mean a DNA of which one of strands codes for the protein when the DNA is double-stranded. The L-homoserine resistance means a property that a bacterium grows on a minimal medium containing L-homoserine at a concentration at which a wild type strain thereof can not grow, usually at 10 mg/ml. The ability to produce an amino acid means a property that a bacterium produces and accumulates the amino acid in a medium in a larger amount than a wild type strain thereof.

15 [0009] According to the present invention, resistance to homoserine of a high concentration can be imparted to a bacterium belonging to the genus *Escherichia*. The cultivation of a bacterium belonging to the genus *Escherichia*, which has increased resistance to homoserine and an ability to accumulate an amino acid, especially, L-homoserine, L-alanine, L-isoleucine, L-valine or L-threonine in a medium results in a high yield of the desired amino acid.

[0010] The present invention will be explained in detail below.

#### (1) DNA of the present invention

[0011] The DNA of the present invention coding for a protein having the Rh activity and having an amino acid sequence shown in SEQ ID NO: 2 in Sequence Listing. Specifically, the DNA of the present invention may be exemplified by a DNA comprising a nucleotide sequence of the nucleotide numbers 557 to 1171 of a nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing.

[0012] The DNA of the present invention includes a DNA fragment encoding the RhtB protein conferring on the bacterium *Escherichia coli* resistance to homoserine, which includes the regulatory elements of the *rhtB* gene and the structural part of *rhtB* gene, having the nucleotide sequence shown in SEQ ID NO: 1.

30 [0013] The nucleotide sequence shown in SEQ ID NO: 1 corresponds to a part of sequence complement to the sequence of GenBank accession number M87049. SEQ ID NO: 1 includes f138 (nucleotide numbers 61959-61543 of GenBank accession number M87049) which is a known but function-unknown ORF (open reading frame) present at 86 min on *E. coli* chromosome, and 5'-flanking and 3'-flanking regions thereof. The f138, which has only 160 nucleotides in the 5'-flanking region, can not impart the resistance to homoserine. No termination codon is present between the 62160 and 61959 of M87049 (upstream the ORF f138). Hence, the coding region is 201 bp longer. Thus the RhtB protein and the *rhtB* gene coding for the protein are novel.

35 [0014] The *rhtB* gene may be obtained, for example, by infecting Mucts lysogenic strain of *E. coli* using a lysate of a lysogenic strain of *E. coli* such as K12 or W3110 according to the method in which mini-Mu d5005 phagemid is used (Groisman, E. A., et al., J. Bacteriol., 168, 357-364 (1986)), and isolating plasmid DNAs from colonies growing on a minimal medium containing kanamycin (40 µg/ml) and L-homoserine (10 mg/ml). As illustrated in the Example described below, the *rhtB* gene was mapped at 86 min on the chromosome of *E. coli*. Therefore, the DNA fragment including the *rhtB* gene may be obtained from the chromosome of *E. coli* by colony hybridization or PCR (polymerase chain reaction, refer to White, T.J. et al, Trends Genet. 5, 185(1989)) using oligonucleotide(s) which has a sequence corresponding to the region hear the portion of 86 min on the chromosome of *E. coli*. Alternatively, the oligonucleotide may be designed according to the nucleotide sequence shown in SEQ ID NO: 1. By using oligonucleotides having nucleotide sequences corresponding to a upstream region from the nucleotide number 557 and a downstream region from the nucleotide number 1171 in SEQ ID NO: 1 as the primers for PCR, the entire coding region can be amplified.

45 [0015] Synthesis of the oligonucleotides can be performed by an ordinary method such as a phosphoramidite method (see Tetrahedron Letters, 22, 1859 (1981)) by using a commercially available DNA synthesizer (for example, DNA Synthesizer Model 380B produced by Applied Biosystems). Further, the PCR can be performed by using a commercially available PCR apparatus (for example, DNA Thermal Cycler Model PJ2000 produced by Takara Shuzo Co., Ltd.), using Taq DNA polymerase (supplied by Takara Shuzo Co., Ltd.) in accordance with a method designated by the supplier.

55 [0016] The DNA coding for the RhtB protein of the present invention may code for RhtB protein including deletion, substitution, insertion, or addition of one or several amino acids at one or a plurality of positions, provided that the Rh activity of RhtB protein encoded thereby is not deteriorated. The DNA, which codes for the substantially same protein as the RhtB protein as described above, may be obtained, for example, by modifying the nucleotide sequence, for example, by means of the site-directed mutagenesis method so that one or more amino acid residues at a specified site

involve deletion, substitution, insertion or addition. DNA modified as described above may be obtained by the conventionally known mutation treatment. The mutation treatment includes a method for treating a DNA coding for the RhtB protein *in vitro*, for example, with hydroxylamine, and a method for treating a microorganism, for example, a bacterium belonging to the genus *Escherichia* harboring a DNA coding for the RhtB protein with ultraviolet irradiation or a mutagenic agent such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and nitrous acid usually used for the mutation treatment.

[0017] The DNA, which codes for substantially the same protein as the RhtB protein, can be obtained by expressing a DNA subjected to *in vitro* mutation treatment as described above in multicopy in an appropriate cell, investigating the resistance to homoserine, and selecting the DNA which increases the resistance. Also, it is generally known that an amino acid sequence of a protein and a nucleotide sequence coding for it may be slightly different between species, strains, mutants or variants, and therefore the DNA, which codes for substantially the same protein, can be obtained from L-homoserine-resistant species, strains, mutants and variants belonging to the genus *Escherichia*. Specifically, the DNA, which codes for substantially the same protein as the RhtB protein, can be obtained by isolating a DNA which hybridizes with DNA having, for example, a nucleotide sequence of the nucleotide numbers 557 to 1171 of the nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing under stringent conditions, and which codes for a protein having the Rh activity, from a bacterium belonging to the genus *Escherichia* which is subjected to mutation treatment, or a spontaneous mutant or a variant of a bacterium belonging to the genus *Escherichia*. The term "stringent conditions" referred to herein is a condition under which so-called specific hybrid is formed, and non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent conditions include a condition under which DNAs having high homology, for example, DNAs having homology of not less than 70% with each other are hybridized, and DNAs having homology lower than the above with each other are not hybridized.

## (2) Bacterium belonging to the genus *Escherichia* of the present invention

[0018] The bacterium belonging to the genus *Escherichia* of the present invention is a bacterium belonging to the genus *Escherichia* of which the Rh activity is enhanced. A bacterium belonging to the genus *Escherichia* is exemplified by *Escherichia coli*. The Rh activity can be enhanced by, for example, amplification of the copy number of the *rhtB* structural gene in a cell, or transformation of a bacterium belonging to the genus *Escherichia* with a recombinant DNA in which a DNA fragment including the *rhtB* structural gene encoding the RhtB protein is ligated with a promoter sequence which functions efficiently in a bacterium belonging to the genus *Escherichia*. The Rh activity can be also enhanced by substitution of the promoter sequence of the *rhtB* gene on a chromosome with a promoter sequence which functions efficiently in a bacterium belonging to the genus *Escherichia*.

[0019] The amplification of the copy number of the *rhtB* structural gene in a cell can be performed by introduction of a multicopy vector which carries the *rhtB* structural gene into a cell of a bacterium belonging to the genus *Escherichia*. Specifically, the copy number can be increased by introduction of a plasmid, a phage or a transposon (Berg, D.E. and Berg, C. M., *Bio/Technol.*, 1, 417 (1983)) which carries the *rhtB* structural gene into a cell of a bacterium belonging to the genus *Escherichia*.

[0020] The multicopy vector is exemplified by plasmid vectors such as pBR322, pMW118, pUC19 or the like, and phage vectors such as  $\lambda$ 1059,  $\lambda$ BF101, M13mp9 or the like. The transposon is exemplified by Mu, Tn10, Tn5 or the like.

[0021] The introduction of a DNA into a bacterium belonging to the genus *Escherichia* can be performed, for example, by a method of D. M. Morrison (*Methods in Enzymology* 68, 326 (1979)) or a method in which recipient bacterial cells are treated with calcium chloride to increase permeability of DNA (Mandel, M. and Higa, A., *J. Mol. Biol.*, 53, 159 (1970)) and the like.

[0022] If the Rh activity is enhanced in an amino acid-producing bacterium belonging to the genus *Escherichia* as described above, a produced amount of the amino acid can be increased. As the bacterium belonging to the genus *Escherichia* in which the Rh activity is to be enhanced, strains which have abilities to produce desired amino acids are used. Besides, the ability to produce an amino acid may be imparted to a bacterium in which the Rh activity is enhanced. Examples of amino acid-producing bacteria belonging to the genus *Escherichia* are described below.

### (1) L-threonine-producing bacteria

[0023] The L-threonine-producing bacteria belonging to the genus *Escherichia* may be exemplified by strain MG442 (Guayatinier et al., *Genetika* (in Russian), 14, 947-956 (1978)).

### (2) L-homoserine-producing bacteria

[0024] The L-homoserine-producing bacteria belonging to the genus *Escherichia* may be exemplified by strain NZ10 (*thrB*). This strain was derived from the known strain C600 (*thrB*, *leuB*) (Appleyard R.K., *Genetics*, 39, 440-452

(1954)) as Leu<sup>+</sup> revertant.

[0025] On the basis of the *rhtB* DNA fragment, new amino acid-producing strains *E. coli* NZ10/pAL4,pRhtB; *E. coli* MG442/pVIC40,pRhtB; and *E. coli* MG442/pRhtB were obtained which are used for the production of amino acids by fermentation.

5 [0026] The new strains have been deposited (according to international deposition based on Budapest Treaty) in the Russian National Collection of Industrial Microorganisms (VKPM) on October 6, 1998. The strain *E. coli* NZ10/pAL4,pRhtB has been deposited as an accession number of VKPM B-7658; the strain *E. coli* MG442/pRhtB has been deposited as an accession number of VKPM B-7659; and the strain *E. coli* MG442/pVIC40,pRhtB has been deposited as an accession number of VKPM B-7660.

10 [0027] The strain *E. coli* NZ10/pAL4,pRhtB (VKPM B-7658) exhibits the following cultural-morphological and biochemical features.

Cytomorphology. Gram-negative weakly-motile rods having rounded ends. Longitudinal size, 1.5 to 2 µm.

15 Cultural features:

[0028]

20 Beef-extract agar. After 24-hour growth at 37°C, produces round whitish semitransparent colonies 1.5 to 3 mm in diameter, featuring a smooth surface, regular or slightly wavy edges, the center is slightly raised, homogeneous structure, pastelike consistency, readily emulsifiable.

Luria's agar. After a 24-hour growth at 37°C, develops whitish semitranslucent colonies 1.5 to 2.5 mm in diameter having a smooth surface, homogeneous structure, pastelike consistency, readily emulsifiable.

25 Minimal agar-doped medium M9. After 40 to 48 hours of growth at 37°C, forms colonies 0.5 to 1.5 mm in diameter, which are colored greyish-white, semitransparent, slightly convex, with a lustrous surface.

Growth in a beef-extract broth. After 24-hour growth at 37°C, exhibits strong uniform cloudiness, has a characteristic odor.

Physiological and biochemical features:

30 [0029]

Grows upon thrust inoculation in a beef-extract agar.

Exhibits good growth throughout the inoculated area.

35 The microorganism proves to be a facultative anaerobe.

It does not liquefy gelatin.

Features a good growth on milk, accompanied by milk coagulation.

Does not produce indole.

40 Temperature conditions. Grows on beef-extract broth at 20-42°C, an optimum temperature lying within 33-37°C.

pH value of culture medium. Grows on liquid media having the pH value from 6 to 8, an optimum value being 7.2.

Carbon sources. Exhibits good growth on glucose, fructose, lactose, mannose, galactose, xylose, glycerol, and mannitol to produce an acid and gas.

Nitrogen sources. Assimilates nitrogen in the form of ammonium, nitric acid salts, as well as from some organic compounds.

45 Resistant to ampicillin, kanamycin and L-homoserine.

L-Threonine is used as a growth factor.

Content of plasmids. The cells contain multicopy hybrid plasmid pAL4 ensuring resistance to ampicillin and carrying the gene *thrA* of the threonine operon, which codes for aspartate kinase-homoserine dehydrogenase I responsible for the increased homoserine biosynthesis. Besides, the cells contain a multicopy hybrid plasmid pRhtB  
50 ensuring resistance to kanamycin and carrying the *rhtB* gene which confers resistance to homoserine (10 mg/l).

[0030] The strain *E. coli* MG442/pRhtB (VKPM B-7659) has the same cultural-morphological and biochemical features as the strain NZ10/pAL4,pRhtB except for L-isoleucine is used as a growth factor instead of L-threonine. However, the strain can grow slowly without isoleucine. Besides, the cells of the strain contain only one multicopy hybrid plasmid pRhtB ensuring resistance to kanamycin and carrying the *rhtB* gene which confers resistance to homoserine (10 mg/l).  
55

[0031] The strain *E. coli* MG442/pVIC40,pRhtB (VKPM B-7660) has the same cultural-morphological and biochemical features as the strain NZ10/pAL4,pRhtB except for L-isoleucine is used as a growth factor instead of L-threonine. However, the strain can grow slowly without isoleucine. The cells of the strain contain multicopy hybrid plasmid pVIC40

ensuring resistance to streptomycin and carrying the genes of the threonine operon. Besides, they contain multicopy hybrid plasmid pRhtB ensuring resistance to kanamycin and carrying the *rhtB* gene which confers resistance to homoserine (10 mg/l).

### (3) Method for producing an amino acid

[0032] An amino acid can be efficiently produced by cultivating the bacterium in which the Rh activity is enhanced by amplifying a copy number of the *rhtB* gene as described above, and which has an ability to produce the amino acid, in a culture medium, producing and accumulating the amino acid in the medium, and recovering the amino acid from the medium. The amino acid is exemplified preferably by L-homoserine, L-alanine, L-isoleucine, L-valine and L-threonine.

[0033] In the method of present invention, the cultivation of the bacterium belonging to the genus *Escherichia*, the collection and purification of amino acid from the liquid medium may be performed in a manner similar to those of the conventional method for producing an amino acid by fermentation using a bacterium. A medium used in cultivation may be either a synthetic medium or a natural medium, so long as the medium includes a carbon and a nitrogen source and minerals and, if necessary, nutrients which the bacterium used requires for growth in appropriate amounts. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids. Depending on assimilatory ability of the used bacterium, alcohol including ethanol and glycerol may be used. As the nitrogen source, ammonia, various ammonium salts such as ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean hydrolyte and digested fermentative microbe are used. As minerals, monopotassium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium carbonate are used.

[0034] The cultivation is preferably culture under an aerobic condition such as a shaking culture, and an aeration and stirring culture. The temperature of culture is usually 20 to 40°C, preferably 30 to 38°C. The pH of the culture is usually between 5 and 9, preferably between 6.5 and 7.2. The pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, a 1 to 3-day cultivation leads to the accumulation of the target amino acid in the medium.

[0035] Recovering the amino acid can be performed by removing solids such as cells from the medium by centrifugation or membrane filtration after cultivation, and then collecting and purifying the target amino acid by ion exchange, concentration and crystalline fraction methods and the like.

### Brief Explanation of Drawing

#### [0036]

Fig. 1 shows cloning, identification and inactivation of the *rhtB* gene.  
Fig. 2 shows the amino acid sequence of the RhtB protein.

### Examples

[0037] The present invention will be more concretely explained below with reference to Examples. In the Examples, an amino acid is of L-configuration unless otherwise noted.

#### Example 1: Obtaining of *rhtB* DNA fragment

##### (1) Cloning of *rhtB* gene into mini-Mu phagemid

[0038] The wild-type *rhtB* gene was cloned *in vivo* using mini-Mu d5005 phagemid (Groisman, E. A., et al., J. Bacteriol., 168, 357-364 (1986)). MuCts62 lysogen of the strain MG442 was used as a donor. Freshly prepared lysates were used to infect a Mucts lysogenic derivative of a strain VKPM B-513 (Hfr K10 metB). The cells were plated on M9 glucose minimal medium with methionine (50 µg/ml), kanamycin (40 µg/ml) and homoserine (10 mg/ml). Colonies which appeared after 48 hr were picked and isolated. Plasmid DNA was isolated and used to transform the strain VKPM B-513 by standard techniques. Transformants were selected on L-broth agar plates with kanamycin as above. Plasmid DNA was isolated from those which were resistant to homoserine, and analyzed by restriction mapping of the structure of the inserted fragments. It appeared that two types of inserts belonging to different chromosome regions had been cloned from the donor. Thus, at least two different genes that is in multicopy and imparts resistance to homoserine exist in *E. coli*. One of the two type of inserts is the *rhtA* gene which has already been reported (ABSTRACTS of 17th International) Congress of Biochemistry and Molecular Biology in conjugation with 1997 Annual Meeting of the American

Society for Biochemistry and Molecular Biology, San Francisco, California August 24-29, 1997). Among the other of the two types of inserts, a fragment of a minimum length which imparts the resistance to homoserine is of 0.8 kb (Fig. 1).

## (2) Identification of *rhtB* gene

[0039] The insert fragment was sequenced by the dideoxy chain termination method of Sanger. Both DNA strands were sequenced in their entirety and all junctions were overlapped. The sequencing showed that the insert fragment included f138 (nucleotide numbers 61543 to 61959 of GenBank accession number M87049) which was a known but function-unknown ORF (open reading frame) present at 86 min of *E. coli* chromosome and 201 bp of the upstream region thereof (downstream region in the sequence of M87049). The f138 which had only 160 nucleotides in the 5'-flanking region could not impart the resistance to homoserine. No termination codon is present upstream the ORF f138 between 62160 and 61959 nucleotides of M87049. Furthermore, one ATG following a sequence predicted as a ribosome binding site is present in the sequence. The larger ORF (nucleotide numbers 62160 to 61546) is designated as *rhtB* gene. The RhtB protein deduced from the gene is highly hydrophobic and contains 5 possible transmembrane segments.

## Example 2: Production of homoserine-producing strain

[0040] Strain NZ10 of *E. coli* was transformed by a plasmid pAL4 which was a pBR322 vector into which the *thrA* gene coding for aspartokinase-homoserine dehydrogenase I was inserted, to obtain the strains NZ10/pAL4. The strain NZ10 is a *leuB*<sup>+</sup>-reverted mutant (*thrB*) obtained from the *E. coli* strain C600 (*thrB*, *leuB*) (Appleyard, Genetics, 39, 440-452 (1954)).

[0041] The *rhtB* gene was inserted to a plasmid pUK21 which is the known plasmid pUC19 in which a kanamycin resistance gene substituted for an ampicillin resistance gene (Vieira, J. and Messing, J., Gene, 100, 189-194 (1991)), to obtain pRhtB.

[0042] The strain NZ10/pAL4 was transformed with pUK21 or pRhtB to obtain strains NZ10/pAL4,pUK21 and NZ10/pAL4,pRhtB.

[0043] The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/l kanamycin and 100 mg/l ampicillin, and 0.3 ml of the obtained culture was inoculated into 3 ml of a fermentation medium having the following composition and containing 50 mg/l kanamycin and 100 mg/l ampicillin, in a 20 x 200 mm test tube, and cultivated at 37°C for 46 hours with a rotary shaker. After the cultivation, an accumulated amount of homoserine in the medium and an absorbance at 560 nm of the medium were determined by known methods.

## Fermentation medium composition (g/L)

[0044]

Glucose	80
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	22
K <sub>2</sub> HPO <sub>4</sub>	2
NaCl	0.8
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.8
FeSO <sub>4</sub> · 7H <sub>2</sub> O	0.02
MnSO <sub>4</sub> · 5H <sub>2</sub> O	0.02
Thiamine hydrochloride	0.0002
Yeast Extract	1.0
CaCO <sub>3</sub>	30
(CaCO <sub>3</sub> was separately sterilized.)	

[0045] The results are shown in Table 1. As shown in Table 1, the strain NZ10/pAL4,pRhtB accumulated homoserine in a larger amount than the strains NZ10/pAL4 and NZ10/pAL4,pUK21 in which the *rhtB* gene was not enhanced.

Table 1

Strain	OD <sub>560</sub>	Accumulated amount of homoserine (g/L)
NZ10/pAL4	16.4	3.1
NZ10/pAL4,pUK21	14.3	3.3
NZ10/pAL4,pRhtB	15.6	6.4

Example 3: Production of alanine, valine and isoleucine with pRhtB-introduced strain

[0046] *E. coli* strain MG442 is a known strain (Gusyatiner, et al., 1978, Genetika (in Russian), 14:947-956).

[0047] The strain MG442 was transformed with the plasmids pUK21 and pRhtB to obtain strains MG442/pUK21 and MG442/pRhtB.

[0048] The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/l kanamycin, and 0.3 ml of the obtained culture was inoculated into 3 ml of the fermentation medium described in Example 3 and containing 50 mg/l kanamycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 40 hours with a rotary shaker. After the cultivation, accumulated amounts of alanine, valine and isoleucine in the medium and an absorbance at 560 nm of the medium were determined by known methods.

[0049] The results are shown in Table 2. As shown in Table 2, the strain MG442/pRhtB accumulated each of alanine, valine and isoleucine in a larger amount than the strain MG442/pUK21 in which the *rhtB* gene was not enhanced.

Table 2

Strain	OD <sub>560</sub>	Accumulated amount (g/L)		
		Alanine	Valine	Isoleucine
MG442/pUK21	13.4	0.2	0.2	0.3
MG442/pRhtB	13.7	0.7	0.5	0.5

Example 4: Production of threonine-producing strain

[0050] The strain MG442 (Example 3) was transformed by introducing a known plasmid pVIC40 (U.S. Patent No. 5,175,107 (1992)) by an ordinary transformation method. Transformants were selected on LB agar plates containing 0.1 mg/ml streptomycin. Thus a novel strain MG442/pVIC40 was obtained.

[0051] The strain MG442/pVIC40 was transformed with pUK21 or pRhtB to obtain strains MG442/pVIC40,pUK21 and MG442/pVIC40,pRhtB.

[0052] The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/l kanamycin and 100 mg/l streptomycin, and 0.3 ml of the obtained culture was inoculated into 3 ml of the fermentation medium described in Example 3 and containing 50 mg/l kanamycin and 100 mg/l streptomycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 46 hours with a rotary shaker. After the cultivation, an accumulated amount of threonine in the medium and an absorbance at 560 nm of the medium were determined by known methods.

[0053] The results are shown in Table 3. As shown in Table 3, the strain MG442/pVIC40,pRhtB accumulated threonine in a larger amount than the strains MG442/pVIC40 and MG442/pVIC40,pUK21 in which the *rhtB* gene was not enhanced.

Table 3

Strain	OD <sub>560</sub>	Accumulated amount of threonine (g/L)
MG442/pVIC40	17	13.6
MG442/pVIC40,pUK21	16.3	12.9



Table 3 (continued)

Strain	OD <sub>560</sub>	Accumulated amount of threonine (g/L)
MG442/pVIC40,pRhtB	15.2	16.3

**Example 5: Effect of *rhtB* gene inactivation and amplification on bacterium *E. coli* resistance to some amino acids and amino acid analogues**

[0054] To inactivate the chromosomal *rhtB* gene the plasmid pNPZ46 was constructed (Fig. 1) on the basis of pUK21 vector. It harbors a DNA fragment from 86 min of *E. coli* chromosome, with the *rhtB* gene and 5'-flanking and 3'-flanking regions thereof. Then the *Clat-Eco47III* fragment of the pNPZ46 plasmid *rhtB* gene was substituted for *AsuII-BsrBI* fragment containing *cat* (Cm<sup>R</sup>) gene of pACYC184 plasmid (Chang and Cohen, J. Bacteriol., 134, 1141-1156, 1978) giving the pNPZ47 plasmid (Fig. 1). To introduce the obtained insertionally inactivated *rhtB* gene into the chromosome of the *E. coli* strain N99 (the streptomycin-resistant derivative of the known strain W3350 (Campbell, Virology, 14, 22-33, 1961)), the method of Parker and Marinus was used (Parker, B. and Marinus, M. G., Gene, 73, 531-535, 1988). The substitution of the wild type allele for the inactivated one was proved by phage P1 transduction and by Southern hybridization (Southern, E. M., J. Mol. Biol., 98, 503-517, 1975).

[0055] Then the susceptibility of the thus obtained *E. coli* strain N99 *rhtB::cat*, of the initial strain N99 (*rhtB*<sup>-</sup>) and of its derivative transformed with pRhtB plasmid, N99/pRhtB, to some amino acids and amino acid analogues was tested. Overnight cultures of the strains grown in M9 minimal medium at 37°C with a rotary shaker (10<sup>9</sup> cfu/ml) were diluted 1:100 and grown for 5 hours under the same conditions. Then the log phase cultures thus obtained were diluted and about 10<sup>4</sup> of alive cells were applied to well-dried test plates with M9 agar containing doubling increments of amino acids or analogues. The minimum inhibitory concentration (MIC) of these compounds were examined after 40-46 h cultivation. The results are shown in Table 4.

Table 4

Substrate	MIC (μg/ml)		
	N99( <i>rhtB</i> <sup>+</sup> )	N99/pRhtB	N99 <i>rhtB::cat</i>
1. L-homoserine	250	30000	125
2. L-threonine	30000	50000	30000
3. L-serine	5000	10000	5000
4. L-valine	0.5	1	0.5
5. AHVA	50	2000	25
6. AEC	10	25	10
7. 4-aza-DL-leucine	40	100	40

[0056] It follows from the Table 4 that multiple copies of *rhtB* besides homoserine conferred upon cells increased resistance to threonine, serine, valine, α-amino-β-hydroxyvaleric-acid (AHVA), S-(2-aminoethyl)-L-cysteine (AEC), and 4-aza-DL-leucine. The inactivation of the *rhtB* gene, on the contrary, increased the cell sensitivity to homoserine and AHVA. These results in conjunction with the data on homology of the RhtB protein to LysE lysine efflux transporter of *Corynebacterium glutamicum* (Vrljic et al., Mol. Microbiol., 22, 815-826, 1996) indicate the analogues function for the *rhtB* gene product. The presumed efflux transporters, RhtB, has specificity to several substrates (amino acids), or may show non-specific effects as a result of amplification.

## SEQUENCE LISTING

<110> Ajinomoto Co., Inc.

<120> DNA coding for protein which confers on bacterium Escherichia coli resistance to L-homoserine, and method for producing L-amino acids

<130>

<140> RU 98118425

<141> 1998-10-13

<160> 2

<170> PatentIn Ver. 2.0

<210> 1

<211> 1200

<212> DNA

<213> Escherichia coli

<220>

<221> CDS

<222> (557)..(1171)

<400> 1

```

agaaataatg tggagatcgc accgcccatac gaatgtgcc a gtatatagcg tttagcggcac 60
ggaccgggct gaacctcctg ctgccagaat gccgccagat catcaacata atcattaaag 120
cgattaacat gcccgagatg cggatcggct aacaggcgac cggaacgtcc ctgcccgcga 180
tggtcgatga ttaagacatc aaaccccaaa tggaaacagg cataggccag ttccgcataat 240
tttagcgtagc tctcaatacg ccccgggcag atgactacca cccggtcag gtgctgtgcg 300
cgaaaacgga caaagcgcac cggaatgtca tccacaccag taaactctgc ttcatacgc 360
tgacgccaga aatcagtcag cgggtcccatg gtaaaagcag caaacgcgtt ttctcttgtt 420
tcccagtctt tttgctgctg aaacatcggg taatctgcct cttaaaccac gtaaaatcgt 480
tttttttagc gtgcctgaca caacgtgcg acagtagcgt attgtggcac aaaaatagac 540
acaccgggag ttcatac atg acc tta gaa tgg tgg ttt gcc tac ctg ctg aca 592
          Met Thr Leu Glu Trp Trp Phe Ala Tyr Leu Leu Thr
          1           5           10
tcg atc att tta acg ctg tcg cca ggc tct ggt gca atc aac act atg 640
Ser Ile Ile Leu Thr Leu Ser Pro Gly Ser Gly Ala Ile Asn Thr Met
          15           20           25
acc acc tcg ctc aac cac ggt tat ccg gcc ggt ggc gtc tat tgc tgg 688
Thr Thr Ser Leu Asn His Gly Tyr Pro Ala Gly Gly Val Tyr Cys Trp
          30           35           40
gct tca gac cgg act ggc gat tca tat tgt gct ggt tgg cgt ggg gtt 736
Ala Ser Asp Arg Thr Gly Asp Ser Tyr Cys Ala Gly Trp Arg Gly Val
          45           50           55           60
ggg acg cta ttt tcc cgc tca gtg att gcg ttt gaa gtg ttg aag tgg 784
Gly Thr Leu Phe Ser Arg Ser Val Ile Ala Phe Glu Val Leu Lys Trp
          65           70           75
gca ggc gcg gct tac ttg att tgg ctg gga atc cag cag tgg cgc gcc 832
Ala Gly Ala Ala Tyr Leu Ile Trp Leu Gly Ile Gln Gln Trp Arg Ala
          80           85           90
gct ggt gca att gac ctt aaa tcg ctg gcc tct act caa tcg cgt cga 880

```

Ala Gly Ala Ile Asp Leu Lys Ser Leu Ala Ser Thr Gln Ser Arg Arg  
 95 100 105  
 5 cat ttg ttc cag cgc gca gtt ttt gtg aat ctc acc aat ccc aaa agt 928  
 His Leu Phe Gln Arg Ala Val Phe Val Asn Leu Thr Asn Pro Lys Ser  
 110 115 120  
 att gtg ttt ctg gcg gcg cta ttt ccg caa ttc atc atg ccg caa cag 976  
 Ile Val Phe Leu Ala Ala Leu Phe Pro Gln Phe Ile Met Pro Gln Gln  
 10 125 130 135 140  
 ccg caa ctg atg cag tat atc gtg ctc ggc gtc acc act att gtg gtc 1024  
 Pro Gln Leu Met Gln Tyr Ile Val Leu Gly Val Thr Thr Ile Val Val  
 145 150 155  
 15 gat att att gtg atg atc ggt tac gcc acc ctt gct caa cgg att gct 1072  
 Asp Ile Ile Val Met Ile Gly Tyr Ala Thr Leu Ala Gln Arg Ile Ala  
 160 165 170  
 cta tgg att aaa gga cca aag cag atg aag gcg ctg aat aag att ttc 1120  
 Leu Trp Ile Lys Gly Pro Lys Gln Met Lys Ala Leu Asn Lys Ile Phe  
 175 180 185  
 20 ggc tcg ttg ttt atg ctg gtg gga gcg ctg tta gca tcg gcg agg cat 1168  
 Gly Ser Leu Phe Met Leu Val Gly Ala Leu Leu Ala Ser Ala Arg His  
 190 195 200  
 gcg tgaaaaataa tgtcggatgc ggcgtaaac 1200  
 25 Ala  
 205  
 <210> 2  
 <211> 205  
 30 <212> PRT  
 <213> Escherichia coli  
 <400> 2  
 Met Thr Leu Glu Trp Trp Phe Ala Tyr Leu Leu Thr Ser Ile Ile Leu  
 1 5 10 15  
 35 Thr Leu Ser Pro Gly Ser Gly Ala Ile Asn Thr Met Thr Thr Ser Leu  
 20 25 30  
 Asn His Gly Tyr Pro Ala Gly Gly Val Tyr Cys Trp Ala Ser Asp Arg  
 35 40 45  
 40 Thr Gly Asp Ser Tyr Cys Ala Gly Trp Arg Gly Val Gly Thr Leu Phe  
 50 55 60  
 Ser Arg Ser Val Ile Ala Phe Glu Val Leu Lys Trp Ala Gly Ala Ala  
 65 70 75 80  
 Tyr Leu Ile Trp Leu Gly Ile Gln Gln Trp Arg Ala Ala Gly Ala Ile  
 85 90 95  
 45 Asp Leu Lys Ser Leu Ala Ser Thr Gln Ser Arg Arg His Leu Phe Gln  
 100 105 110  
 Arg Ala Val Phe Val Asn Leu Thr Asn Pro Lys Ser Ile Val Phe Leu  
 115 120 125  
 50 Ala Ala Leu Phe Pro Gln Phe Ile Met Pro Gln Gln Pro Gln Leu Met  
 130 135 140  
 Gln Tyr Ile Val Leu Gly Val Thr Thr Ile Val Val Asp Ile Ile Val

145                      150                      155                      160  
 Met Ile Gly Tyr Ala Thr Leu Ala Gln Arg Ile Ala Leu Trp Ile Lys  
 5                      165                      170                      175  
 Gly Pro Lys Gln Met Lys Ala Leu Asn Lys Ile Phe Gly Ser Leu Phe  
 180                      185                      190  
 Met Leu Val Gly Ala Leu Leu Ala Ser Ala Arg His Ala  
 10                      195                      200                      205

# 15 Claims

1. A protein comprising an amino acid sequence shown in SEQ ID No. 2 of the Sequence Listing or including deletion, substitution, insertion and/or addition of one or more amino acids in said sequence and having the activity of making a bacterium resistant to L-homoserine.
2. A DNA sequence coding for a protein according to claim 1.
3. The DNA sequence according to claim 2 having the sequence of nucleotide numbers 557 to 1171 of the sequence shown in SEQ ID No. 1 of the Sequence Listing.
4. The DNA sequence according to claim 2 which hybridizes with the sequence of nucleotide numbers 557 to 1171 of the sequence shown in SEQ ID No. 1 of the Sequence Listing under stringent conditions.
5. A vector containing the DNA sequence according to any of the claims 2 to 4.
6. A bacterium, wherein resistance to L-homoserine is enhanced by increasing the concentration of the protein according to claim 1 in the cell of said bacterium.
7. The bacterium according to claim 6, wherein the expression rate of the gene containing the DNA sequence according to any of the claims 2 to 4 is increased and/or the copy number of the DNA sequence according to any of the claims 2 to 4 is amplified.
8. The bacterium according to claim 7, wherein said DNA is carried on a transposon.
9. A bacterium containing the vector according to claim 5.
10. A method for producing an amino acid comprising the step of cultivating the bacterium according to any of the claims 6 to 9 having the ability to produce the amino acid, in a culture medium, and producing and accumulating the amino acid in the medium.
11. The method according to claim 10, wherein said amino acid is at least one selected from the group consisting of L-homoserine, L-alanine, L-isoleucine, L-valine and L-threonine.

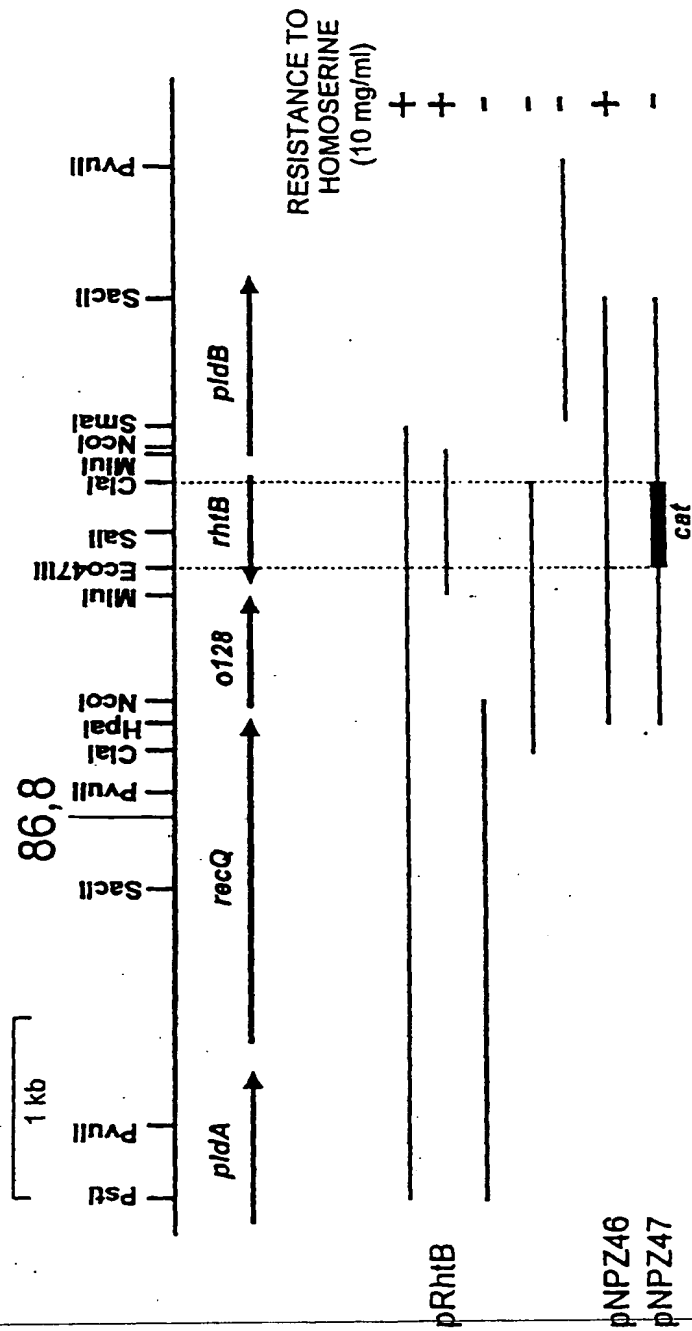


FIG. 1

Met	Thr	Leu	Glu	Trp	Trp	Phe	Ala	Tyr	Leu	Leu	Thr	Ser	Ile	Ile	Leu
1				5					10					15	
Thr	Leu	Ser	Pro	Gly	Ser	Gly	Ala	Ile	Asn	Thr	Met	Thr	Thr	Ser	Leu
			20					25					30		
Asn	His	Gly	Tyr	Pro	Ala	Gly	Gly	Val	Tyr	Cys	Trp	Ala	Ser	Asp	Arg
		35				40						45			
Thr	Gly	Asp	Ser	Tyr	Cys	Ala	Gly	Trp	Arg	Gly	Val	Gly	Thr	Leu	Phe
	50					55					60				
Ser	Arg	Ser	Val	Ile	Ala	Phe	Glu	Val	Leu	Lys	Trp	Ala	Gly	Ala	Ala
	65				70					75					80
Tyr	Leu	Ile	Trp	Leu	Gly	Ile	Gln	Gln	Trp	Arg	Ala	Ala	Gly	Ala	Ile
				85					90					95	
Asp	Leu	Lys	Ser	Leu	Ala	Ser	Thr	Gln	Ser	Arg	Arg	His	Leu	Phe	Gln
			100					105					110		
Arg	Ala	Val	Phe	Val	Asn	Leu	Thr	Asn	Pro	Lys	Ser	Ile	Val	Phe	Leu
		115					120					125			
Ala	Ala	Leu	Phe	Pro	Gln	Phe	Ile	Met	Pro	Gln	Gln	Pro	Gln	Leu	Met
	130					135					140				
Gln	Tyr	Ile	Val	Leu	Gly	Val	Thr	Thr	Ile	Val	Val	Asp	Ile	Ile	Val
	145				150					155					160
Met	Ile	Gly	Tyr	Ala	Thr	Leu	Ala	Gln	Arg	Ile	Ala	Leu	Trp	Ile	Lys
			165					170					175		
Gly	Pro	Lys	Gln	Met	Lys	Ala	Leu	Asn	Lys	Ile	Phe	Gly	Ser	Leu	Phe
			180					185					190		
Met	Leu	Val	Gly	Ala	Leu	Leu	Ala	Ser	Ala	Arg	His	Ala			
		195					200					205			

(SEQ ID NO: 2)

FIG. 2

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER: \_\_\_\_\_**

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**